III. Conclusion

The present document is a full and complete response to the Action. In conclusion, Applicants submit that, in light of the foregoing remarks, the present case is in condition for allowance, and such favorable action is respectfully requested. Should Examiner Li have any questions or comments, or believe that certain amendments of the claims might serve to improve their clarity, a telephone call to the undersigned Applicants' representative is earnestly solicited.

Respectfully submitted,

Peter G Seferian Reg No 49962

July 2, 2004
Date

Lance K Ishimoto Reg. No. 41,866

LEXICON GENETICS INCORPORATED
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Customer # 24231

App Serial # 09/735,712 Exhibit A
Walke et al. LEX-0109-USA
Novel Human Membrane Proteins and Polynucleotides Encoding the Same

*606499 MEMBRANE-SPANNING 4-DOMAINS, SUBFAMILY A, MEMBER 5; MS4A5

Alternative titles; symbols

TESTIS-EXPRESSED TRANSMEMBRANE 4; TETM4
CD20 ANTIGEN-LIKE 2; CD20L2

Gene map locus 11q12-q13

TEXT

MS4A family proteins share structural similarity, amino acid sequence homology, and chromosomal location. They contain 4 highly conserved transmembrane domains, flanked by N- and C-terminal cytoplasmic regions.

CLONING

By EST database searching for sequences homologous to a conserved sequence in the second transmembrane region of CD20 (MS4A1; 112210), followed by 3-prime RACE, Hulett et al. (2001) isolated an MS4A5 cDNA, which they called TETM4. The deduced 200-amino acid protein shares 20 to 30% sequence identity with other members of the MS4A family, including CD20, FCER1B (MS4A2; 147138), and HTM4 (MS4A3; 606498). MS4A5 has a cytoplasmic N terminus, 4 transmembrane domains, 2 extracellular loops, 1 intracellular loop, and a cytoplasmic C terminus. Northern blot and RT-PCR analysis detected expression of a 0.7-kb transcript restricted to testis, distinct from the hematopoietic tissue expression of other MS4A proteins.

Independently, Liang and Tedder (2001) and Ishibashi et al. (2001) obtained MS4A5 cDNAs and detected expression in testis.

MAPPING

By FISH and radiation hybrid analysis, Hulett et al. (2001) mapped the MS4A5 gene to chromosome 11q12-q13 in a cluster with MS4A1, MS4A2, and MS4A3.

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Isolation, tissue distribution, and chromosomal localization of a novel testis-specific human four-transmembrane gene related to CD20 and Fc-epsilon-RI-beta. *Biochem. Biophys. Res. Commun.* 280: 374-379, 2001.
PubMed ID : 11162526

2. Ishibashi, K.; Suzuki, M.; Sasaki, S.; Imai, M. :
Identification of a new multigene four transmembrane family (MS4A) related to CD20, HTm4 and beta subunit of the high affinity IgE receptor. *Gene* 264: 87-93, 2001.
PubMed ID : 11245982

3. Liang, Y.; Tedder, T. F. :
Identification of a CD20-, Fc-epsilon-RI-beta-, and HTm4-related gene family: sixteen new MS4A family members expressed in human and mouse. *Genomics* 72: 119-127, 2001.
PubMed ID : 11401424

CREATION DATE

Paul J. Converse : 11/26/2001

EDIT HISTORY

carol : 11/26/2001

App Serial # 09/735,712 Exhibit B
Walke et al. LEX-0109-USA
Novel Human Membrane Proteins and Polynucleotides Encoding the Same

CD20/IgE Fc receptor beta subunit

Accession	**IPR007237;** (CD20) matches 43 proteins
FullName	CD20/IgE Fc receptor beta subunit
Type	Family
Signatures	PFAM: PF04103 *CD20*
Molecular Function	receptor activity (GO:0004872)
Biological Process	signal transduction (GO:0007165)
Cellular Component	integral to membrane (GO:0016021)
Abstract	This family includes the CD20 protein and the beta subunit of the high affinity receptor for IgE Fc. The high affinity receptor for IgE is a tetrameric structure consisting of a single IgE-binding alpha subunit, a single beta subunit, and two disulphide-linked gamma subunits. The alpha subunit of Fc epsilon RI and most Fc receptors are homologous members of the Ig superfamily. By contrast, the beta and gamma subunits from Fc epsilon RI are not homologous to the Ig superfamily. Both molecules have four putative transmembrane segments and a probable topology where both N- and C termini protrude into the cytoplasm [1].
Examples	• P19437 • Q01362 • P20490 View Signature matches on the examples
References	1. Hupp,K., Siwarski,D., Mock,B.A., Kinet,J.P., Gene mapping of the three subunits of the high affinity FcR for IgE to mouse chromosomes 1 and 19. (1989) *J. Immunol.* **143**: 3787-3791 [PUBMED:2531187] [PUB00009834]
Database links	Transport:1.A.37.1.1 ;
Taxonomy Distribution	Chordata:43 Eukaryota:43 Human:16

(links to UniProt))	Metazoa:43 Mouse:24
Matches	Table all Graphical all

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	D.Wade Walke	Group Art Unit:	1646
Application No.:	09/735,712	Examiner:	R. Li
Filed:	12/02/2000		
Title:	Novel Human Membrane Proteins and Polynucleotides Encoding the Same	Atty. Docket No.	LEX-0109-USA

DECLARATION OF TAMAS ORAVECZ UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Alexandria, VA 22313

Sir:

I, TAMAS ORAVECZ, declare that:

1. I am a citizen of the Hungary and reside at 34 Endor Forest Place, The Woodlands, Texas, 77382.

2. I am currently the Director of Immunology at Lexicon Genetics Incorporated, The Woodlands, Texas, the assignee of the above-identified application, where I have been employed since 2000. I received the degree of Doctor of Philosophy from the University of Szeged, Hungary in 1991, where I studied the molecular interactions and signal transduction pathways involved in cell signaling in the immune system. Prior to joining Lexicon Genetics Incorporated, I headed the HIV Therapy Program at SyStemix Incorporated – Novartis, Cell and Gene Therapy, located in Palo Alto, CA, where I developed and evaluated new strategies to inhibit HIV infection of immune cells and investigated the mechanism of action of novel pharmaceutical compounds with antiviral activity. Prior to that position I was a Senior Scientist and Primary Reviewer in the Laboratory of Cell and Viral Regulation at the Center for Biologics Evaluation and Research at the National Institutes of Health and Food and Drug Administration in Bethesda, MD. My technical experience and publications are summarized in my *Curriculum Vitae*, which is appended hereto as Exhibit 1.

3. I have reviewed the above-identified patent application and the Final Office Action mailed on October 28, 2003. I understand that the Examiner has rejected claims 1-9 under 35 U.S.C. § 101, on the grounds that the claimed invention lacks either a specific, substantial, and credible utility or a well-established utility. In addition, the Examiner has also rejected claims 1-9 under 35 U.S.C. § 112, first paragraph, on the grounds that one skilled in the art would not know how to use the claimed invention, as the invention allegedly is not supported by a specific, substantial, and credible utility or a well-established utility.

4. I am providing the present declaration to present evidence that the murine ortholog of the human CD20 antigen-like membrane proteins encoded by the sequences of the present invention have an effect on natural killer (NK) cell levels *in vivo* and that NK cell levels are known by those of skill in the art to be involved in the pathophysiology of connective tissue disorders, such as systemic sclerosis, in humans.

5. It is my understanding that the present application claims human sequences that encode isoforms of a CD20 like protein known as Membrane-Spanning 4-domains subfamily A member 5 (MS4A5). Genetically engineered transgenic "knockout" mice in which the gene that encodes the murine ortholog of the claimed human sequence was disrupted were created in order to determine the function *in vivo* of the mouse protein encoded by the murine ortholog of the claimed human sequences. These knockout mice were subject to a medical work-up using an integrated suite of medical diagnostic procedures designed to assess the function of the major organ systems in a mammalian subject. I supervised the analysis of lymphoid cell phenotypes using standard labeled antibody and flow cytometric methods. During this analysis the level of natural killer (NK) cells present in blood samples obtained from homozygous (-/-) "knock-out" mice, those in which both copies of the murine ortholog of the claimed human sequences were disrupted, were compared to blood samples obtained from wild-type (+/+) mice, those in which the murine ortholog of the claimed human sequences was fully functional.

6. The results of this analysis indicates that disruption of the murine ortholog of the claimed human sequences of the present invention results in an increase in the mean

percentage of NK cells present in the blood of homozygous (-/-) "knock-out" mice as compared to wild-type (+/+) littermate controls. The mean percentage of NK cells present in the two wild-type (+/+) mice tested was 4.8 ± 0.6 %. The mean percentage of NK cells present in the four homozygous (-/-) "knock-out" mice tested was 8.4 ± 1.6 %. The increased level of NK cells observed following disruption of the murine ortholog of the claimed human sequences indicates that the mouse protein encoded by the murine ortholog of the claimed human sequences has an effect on the level of NK cells present in the blood *in vivo*.

7. Additional flow cytometric analyses performed on additional animals, five wild-type (+/+) mice and five homozygous (-/-) "knock-out" mice, confirmed that disruption of the murine ortholog of the protein of the claimed human sequences results in an increase in the mean level of NK cells present in the immune system of homozygous (-/-) "knock-out" mice as compared to wild-type (+/+) littermate controls. The mean percentage of NK cells present in the spleen of the five additional wild-type (+/+) mice tested was 6.85 ± 1.06 %. The mean percentage of NK cells present in the spleen of the five additional homozygous (-/-) "knock-out" mice tested was 9.45 ± 0.72 %. This difference was statistically significant ($p=0.002$). The increased level of NK cells observed following disruption of the murine ortholog of the claimed human sequences in these additional mice confirms earlier findings that the protein encoded by the murine ortholog of the claimed human sequences has an effect on the level of NK cells present in the immune system *in vivo*.

8. It is my opinion that *in vivo* findings obtained in the mouse are widely considered by those of skill in the art to be predictive of findings in the human. Amongst many other forms of evidence, including the acceptance by the U.S. Food and Drug Administration, this position is supported by a recent retrospective study in which it was determined that most of the human drug targets to which recently approved human therapies are directed could have been predicted using transgenic "knockout" mice, mice in which the murine ortholog of the human sequence had been disrupted. This publication is presented in Exhibit 2. Thus, I am of the opinion that the *in vivo* data obtained in mice by disrupting the murine ortholog of the claimed human sequences indicates that the product encoded by the sequences of the present application would also be expected to modulate NK cell levels in

humans.

9. It is also my opinion that those of skill in the art are aware of the association between natural killer (NK) cells and the pathophysiology of connective tissue disorders, such as systemic sclerosis. This is evidenced in the abstracts of the peer reviewed publications provided in Exhibit 3.

10. Given the evidence presented in this declaration, I am of the opinion that in light of the *in vivo* findings in the mouse, that those skilled in art would readily believe that the human CD20-like protein encoded by the claimed human sequences also plays a role in the regulation of NK cell levels and that altered NK cell levels are associated with connective tissue disorders. Therefore, those skilled in the art would have no reason not to believe that the CD20-like protein of the present invention is associated with NK cell levels and thus with connective tissue disorders.

11. I declare further that all statements made in this Declaration are of my own knowledge and are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 6/28/04

TAMAS ORAVECZ, Ph.D.

TAMAS ORAVECZ, PH.D.

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SUMMARY OF SKILLS

- Expertise in immunology and virology.
- Extensive 10+ years experience in research and development.
- Directed diverse, concurrent projects from experimental research phase to clinical trials.
- Excellent written/verbal communication skills, proven leadership and managerial skills, good publication record.
- Solid working knowledge in the development of a wide range of techniques in molecular and cellular immunology and in virology.
- Experience in Drug Approval Process and regulations, writing of IND and patent applications.

PROFESSIONAL EXPERIENCE

2000-present Director, Immunology/Hematology
Lexicon Genetics, Inc., The Woodlands, TX

Program: Identify and validate targets for drug discovery by using *in vivo* functional genomics technology.

- Design and implement strategies for large-scale evaluation of gene function. Define target areas of interest for small molecule and biotherapeutic drug discovery and provide expertise on identifying new candidate drug targets and pathways, and extracting meaningful biological information on gene function. Establish and manage projects with potential value for novel pathway and drug target discovery.
- Manage development of high throughput assays, lead compound identification, and pre-clinical evaluation.
- Responsible for building up the Immunology/Hematology Department at the company, introducing state of the art technologies, and directing the research and development in the department.
- Make recommendations on scientific collaborations, partnerships, patenting and licensing issues. Provide expertise in immunology and virology, and participate in the corporate level decision making process. Report directly to the Senior Vice President of Pharmaceutical Biology.

1998-2000 Program Head
HIV Therapy
SyStemix, Inc. – Novartis Cell and Gene Therapy, Palo Alto, CA

Program: Develop and evaluate new strategies to inhibit HIV infection of immune cells. Investigate mechanism of action of novel pharmaceutical compounds with antiviral activity.

- Managed diverse, concurrent internal projects in HIV therapy from experimental research phase to clinical trials.
- Activated productive external collaborations relevant to success of projects.
- Developed strategies that capitalize on key scientific opportunities and serve to maintain proprietary position.
- Evaluated novel technologies and make recommendations on licensing.
- Provided expertise as research liaison of the International Project Team Management for HIV Therapy.

1996-1998 Senior Scientist / Primary Reviewer
1991-1996 Postdoctoral Fellow

Laboratory of Cell and Viral Regulation, Center for Biologics Evaluation and Research, National Institutes of Health / Food and Drug Administration, Bethesda, MD.

Research

Investigated the structure and function of leukocyte surface receptors and chemokines, and the signaling mechanisms responsible for immunological dysfunction after HIV-1 infection:

- Defined the functional importance of different CD4 epitopes and HIV-1 envelope glycoproteins in modifying T-cell responses.
- Described the mechanism of CC-chemokine suppression of HIV-1 infection.
- Identified the RANTES chemokine as the first natural substrate of the dipeptidyl-peptidase IV enzyme, CD26.
- Demonstrated that cell surface proteoglycans contribute to chemokine antiviral activity and to HIV-1 infection.
- Made monoclonal antibodies against cell surface markers with novel specificity. Chaired sessions in International Workshops on Leukocyte Differentiation Antigens.

Review and management

- Participated in the regulatory work of the U.S. Food and Drug Administration. Reviewed Investigational New Drug (IND) Applications.
- Trained post-doctoral fellows and research associates, and supervised their performance.

1988-1991	Research Fellow, Laboratory of Molecular Immunology, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

Studies on the molecular interactions and signal transduction pathways involved in cell signaling in the immune system:

- Described the co-stimulatory function of the protein-tyrosine-phosphatase CD45 molecules in T-cell activation.
- Characterized novel disulfide-bound molecular structures involving CD45.
- Studied the immunohistochemical localization of Raf protein kinase.
- Participated in the characterization of monoclonal antibodies analyzed by the Fourth International Workshop on Leukocyte Differentiation Antigens.
- Taught courses on Molecular Immunology. Wrote book-chapter on the analysis of cell surface receptors for medical students and researchers in immunology.

EDUCATION

1983-1988	M.S., Molecular Biology/Biotechnology, University of Szeged, Hungary
1991	Ph.D., General Physiology and Molecular Biology, University of Szeged, Hungary
1995	The American Association of Immunologists Advanced Course in Immunology, Berkeley, California
1997	Reviewer Training Course, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Rockville, MD

FELLOWSHIPS AND AWARDS

1989	Fellowship of the Swiss Society for Immunology, Lausanne, Switzerland
1990	Fellowship of the European Association of Immunologists, Toulon, France
1991	Fellowship of the British Council, Dr. Peter C.L. Beverley, Imperial Cancer Research Fund, London, England
1991-1996	Fellowship of the Fogarty International Center, National Institutes of Health, Bethesda, MD, USA
1996	Travel Award, 6th International Conference on Human Leukocyte Differentiation Antigens, Kobe, Japan
1997	Science Recognition Award for New Investigators, Clinical Immunology Society, San Francisco, CA, USA

Issued Patent

T. Oravecz and M.A. Norcross. Chemokine variants and methods of use. WO9928474

Memberships

American Association of Immunologists
Clinical Immunology Society
American Society for Microbiology

Service to Professional Publications

The Journal of Immunology	Primary reviewer
The Journal of Infectious Diseases	Ad-hoc reviewer
Journal of Leukocyte Biology	Ad-hoc reviewer

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H. Belrandelrio, F. Kern, T. Lanthorn, T. Oravecz, J. Piggott, D. Powell, R. Ramirez-Solis, A.T. Sands and B. Zambrowicz

KNOCKOUTS MODEL THE 100 BEST-SELLING DRUGS — WILL THEY MODEL THE NEXT 100?

Brian P. Zambrowicz and Arthur T. Sands

The biopharmaceutical industry is currently faced with a tremendous number of potential drug targets identified through the sequencing of the human genome. The challenge ahead is to delineate those targets with the greatest value for therapeutic intervention. Here, we critically evaluate mouse-knockout technology for target discovery and validation. A retrospective evaluation of the knockout phenotypes for the targets of the 100 best-selling drugs indicates that these phenotypes correlate well with known drug efficacy, illuminating a productive path forward for discovering future drug targets. Prospective mining of the druggable genome is being catalysed by large-scale mouse knockout programs combined with phenotypic screens focused on identifying targets that modulate mammalian physiology in a therapeutically relevant manner.

The sequencing of the human genome is catalysing a transformation in drug discovery by offering an unprecedented opportunity for the development of novel therapeutics. Never before has there been access to the code for all human genes and potentially all host targets for pharmaceutical development. Some have estimated there may be as many as 5,000–10,000 new drug targets within the genome[1], and it is not uncommon to hear therapeutic discovery groups comment that they have more targets than they know what to do with. However, are such expansive estimates supported by scientific experience or does a proliferation of non-validated targets threaten to clog screening pipelines globally, drive up research and development costs, and degrade industry productivity? The challenge ahead is to efficiently translate the huge discovery potential of the genome into real products. Now is a good time to critically assess what can be learned from reverse genetic studies of the current targets of the pharmaceutical industry in order to determine the best steps for moving forward with a sound discovery strategy in the post-genomic era. In this review, we evaluate the data from mouse knockouts (KOs) of the targets of the 100 best-selling drugs to establish a retrospective view of the success of genetic antagonism to model therapeutic intervention in the mammal. The significance of new high-quality targets is considered in the context of how many targets are currently responsible for revenue from the 100 best-selling drugs, and the rarity and value of commercializing truly novel targets. We discuss our ongoing use of large-scale, reverse mouse genetics to discover those genes, among thousands of sequences, that encode truly valuable new targets for pharmaceutical development. Finally, our creation and phenotypic analysis of the physiological functions of more than 750 novel gene KOs allows us to make predictions as to the number of biologically validated, therapeutically relevant drug targets we can realistically expect to discover over the next five years from the human genome.

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doi:10.1038/nrd987

Target base and new target innovation

It has been suggested that, historically, all drugs have addressed a total of approximately 500 molecular targets[1]. This represents a significant number of targets, but does not indicate how many targets provide ongoing commercial value. A recent review identified only 120 targets for all marketed drugs[2]. Similarly, a survey of prescription drugs marketed by the top ten

pharmaceutical companies reveals that fewer than 100 targets are responsible for all prescription products marketed. A more meaningful view of targets can be obtained by looking at the top-selling drugs of the industry. The 100 best-selling drugs of 2001 are directed at only 43 host proteins, which highlights that the economics of the pharmaceutical industry actually relies on a small pool of targets[3].

Another important parameter for drug discovery is the rate at which new targets are successfully commercialized by the pharmaceutical industry. An examination of the new drugs approved over the past nine years gives an indication of the productivity and innovation related to the development of new targets. The most common way to quantify productivity is to look at the number of new molecular entities (NMEs) that are approved each year by the US Food and Drug Administration (FDA) (FIG. 1)[4–6]. An NME is defined by the FDA as an active ingredient that has never been marketed in the United States. Over the past nine years, an average of 31 NMEs have been approved each year, with a high of 53 in 1996 and a low of 18 in 1994. The number of NMEs has not varied much over the past nine years, although there has been a downward trend over the past couple of years.

Although a look at the number of NMEs approved each year gives some indication of levels of innovation, it does not provide an accurate account of the number of new targets that are commercialized by the pharmaceutical industry each year. A critical assessment of the targets of NMEs reveals that most of the NMEs are 'me too' drugs — that is, drugs that modulate targets for which there are already drugs on the market. For example, in the year 2001, the 24 NMEs approved included additional drugs that modulate cyclooxygenase 2 (COX2), the serotonin $5HT_3$ receptor, β_2-adrenoceptor, histamine H_1 receptor, Factor X and acetyl cholinesterase[4]. In fact, a surprisingly small number of truly innovative host targets or therapeutic proteins — typically two to three — are commercialized each year by the entire pharmaceutical industry (FIG. 1 and TABLE 1).

Although this small number is sobering, the bright side is that these innovator targets provide tremendous potential for the industry. Breakthrough targets deliver completely new mechanisms for treating disease and can therefore rapidly create large new medical markets. Several examples of breakthrough targets include COX2, phosphodiesterase type 5 (PDE5) and BCR-ABL. The COX2 target was first commercialized in 1998 with the approval of celecoxib (Celebrex) for arthritis, which generated US $1 billion in sales within one year of approval. Sildenafil (Viagra) was also brought to market in 1998 for the treatment of erectile disfunction and achieved sales of US $1.5 billion in 2001. These breakthrough targets are not just commercial successes, but also provide hope for the treatment of unmet medical needs. In 2001, the revolutionary drug imatinib mesylate (Gleevec) was approved. Gleevec inhibits the *BCR-ABL* oncogene and has been a significant breakthrough for the treatment of CHRONIC MYELOID LEUKEMIA (CML). Treatment with imatinib mesylate has resulted in haematological and cytogenetic remissions in most patients with chronic-phase CML[7]. These examples indicate that although rare, these high-quality breakthrough targets are exceptionally valuable and provide hope for the treatment of unmet medical needs.

CHRONIC MYELOID LEUKAEMIA
A leukaemia characterized by the presence of large numbers of abnormal mature granulocytes circulating in the blood.

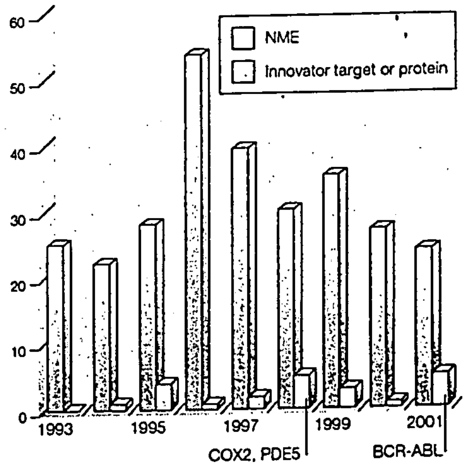

Figure 1 | **Worldwide biopharmaceutical productivity: new molecular entities versus breakthrough or innovator targets.** This graph shows the number of new molecular entities (NMEs) approved by the US Food and Drug Administration (FDA) each year over the past nine years, and compares this with the number of breakthrough host targets that are commercialized each year. Three examples of breakthrough targets and the years that they were approved are indicated. COX2, cyclooxygenase 2; PDE5, phosphodiesterase type 5.

Given that the pharmaceutical industry is reliant on fewer than 100 targets and commercializes only two to three new targets per year, it might be worth reassessing what genomics can realistically offer drug discovery in terms of the identification of novel targets. Historic data suggest that the industry has relied, and might well continue to rely, on a relatively small number of targets. We have also collected genetic data (to be discussed later) that indicate that the number of high-quality targets from the genome may not be as large as some have predicted. If the number of targets is limited, then high-quality targets are more like needles in a genomic haystack than abundant low-hanging fruit for the picking. The technologies required for finding these targets should not simply provide a huge number of potential candidates, but must instead provide a strong filter for determining the physiological significance of targets, therefore allowing drug-discovery efforts to focus on only the most promising targets.

Genetics works

The goal of genomics-based drug discovery is to translate gene-sequence data and discoveries into drugs that provide defined physiological and clinical endpoints. The challenge in genomics has been to define a consistent and efficient process for moving from sequence data to drug entity. The confusion in

Table 1 | **FDA-accepted breakthrough or innovator targets***

Year	Drug	Innovator target
1994	Glucophage	Perhaps acetyl CoA carboxylase 2
1995	Precose	α-glucosidase
1995	Cozaar	Angiotensin receptor AT_1
1995	CellCept	Inosine monophosphate dehydrogenase
1995	Fosamax	Perhaps farnesyl diphosphate synthase
1996	Accolate	Leukotriene receptor
1997	Plavix	Platelet $P2Y_{12}$ receptor
1997	Rezulin	Peroxisome proliferator activated receptor
1998	Celebrex	Cyclooxygenase 2
1998	Aggrastat Integrilin	Platelet glycoprotein IIb/IIIa receptor
1998	Viagra	Phosphodiesterase type 5
1998	Enbrel Remicade	Recombinant receptor or antibody to bind tumour necrosis factor α
1998	Herceptin	ERBB2 (also known as HER2/neu)
1999	Rapamune	FK-binding protein 12 and target of rapamycin (TOR kinase)
1999	Xenical	Gastrointestinal lipase
1999	Targretin	Retinoid X receptors
2000	Mylotarg	Antibody to CD33
2001	Gleevec	BCR-ABL
2001	Tracleer	Endothelin receptor
2001	Natrecor	Recombinant B-type natriuretic peptide
2001	Xigris	Recombinant activated protein C
2001	Kineret	Recombinant interleukin 1 receptor antagonist

*Breakthrough or innovator targets accepted by the US Food and Drug Administration (FDA) over the past nine years. This information was compiled on the basis of the FDA Center for Drug Evaluation and Research Reports to the Nation 1993–2001 and Drug Topics Archives, New Drug Approvals 1995–2001.

the field has been exemplified by the coining of various catch phrases for the 'next best technology', including functional genomics, pharmacogenomics, proteomics and METABOLOMICS, to name a few of the many ever-proliferating -omics disciplines. The question remains as to what technologies might allow one to cut through the confusion in the industry and provide consistent value in drug discovery. Mouse genetics has become a powerful approach for defining gene function in the context of mammalian physiology[8,9]. However, questions concerning the value of mouse genetics for drug discovery are common and varied: What is the correlation between mouse and human physiology? What is the relevance of a KO phenotype to developing a small-molecule drug? Does gene compensation prevent one from identifying the true function of genes? How is a KO throughout development relevant to what a gene does in the adult? And does embryonic lethality prevent the identification of many of the best targets? To address the questions above in an objective manner, we have chosen the 100 best-selling drugs of 2001, identified their targets and compiled the KO phenotypes for those targets. These are the targets that are crucial to the US $148 billion of prescription drug sales in the United States alone in 2001 (REF. 10).

METABOLOMICS
The quantitative measurement of all low-molecular-weight metabolites in an organism's cells at a specified time under specific environmental conditions.

GASTROESOPHAGEAL REFLUX DISEASE
A disorder in which there is recurrent return of stomach contents back up into the oesophagus, frequently causing heartburn, a symptom of irritation of the oesophagus by stomach acid.

The results of the analysis present a compelling case for the power of gene-knockout technology to describe the action of blockbuster drugs (summarized in ONLINE TABLE 8). The 100 best-selling drugs modulate roughly 43 host targets, which represents approximately 50% of all host targets for which marketed drugs exist. The remaining 14 drugs are anti-infectives with non-host targets, making them unamenable to KO mouse analysis. In general, the targets of the top 100 pharmaceutical drugs are not human genes that directly cause disease, but are key biochemical switches that produce a desirable change in the physiological state of the organism, which in turn alter or abrogate an ongoing disease process. This emphasizes the strategy of identifying key switches in mammalian physiology that can be modulated to provide a therapeutic effect, which is very different from trying to identify human disease genes that may not themselves be amenable to therapeutic intervention. Of the 43 mammalian targets, 34 have been knocked out and 29 of the resulting KO phenotypes have been informative in terms of illuminating gene function and pharmaceutical utility, and providing, in most cases, a direct correlation between KO phenotype and the therapeutic effect of the drug. Overall, concerns about mutations that operate throughout development, gene compensation, embryonic lethality and the differences between mouse and human physiology have not been an issue for the most important targets in the pharmaceutical industry. What follows is a summary of the phenotypes identified on the basis of the KOs of the targets for the 100 best-selling drugs, which have been broken down into their respective areas of therapeutic utility. A full table giving correlations between the 100 best-selling drugs and their KO phenotypes is provided as an online-only feature of the article (ONLINE TABLE 8). Where KO animals exist, this information is also given in print, broken down and grouped according to disease indication.

Gastroesophageal reflux disease

The proton-pump inhibitors used to treat GASTROESOPHAGEAL REFLUX DISEASE (GERD) (for example, omeprazole (Prilosec), lansoprazole (Prevacid and Takepron) and pantoprazole (Pantozol)) target the hydrogen/potassium ATPase in order to lower gastric-acid secretion. This target comprises two subunits — the α and β subunits — that are encoded by separate genes, and which can therefore be knocked out independently. The gastric contents of the α-subunit-KO mice were examined after histamine treatment[11]. The pH of the stomach contents for α-subunit-null mice was 6.9, compared with a pH of 3.17 for the wild-type controls. The KO mice also displayed histopathological abnormalities that included hyperplasia and disruption of the architecture of the gastric glands (TABLE 2). Similarly, KO of the β subunit resulted in animals with achlorhydric stomach contents, with a pH of 7 relative to a pH of 3.6 for wild-type controls. β-subunit KOs also showed histopathological alterations of the stomach, specifically with respect to parietal cells[12]. KO of the α or β subunit of the proton pump results in a phenotype that is exactly what one might expect based upon the known activity of the pharmacological inhibitors.

Table 2 | **Best-selling drugs and the KO-mouse phenotype: GERD and haematopoietic disorders***

Drug target	Drug name (utility)	2001 Sales‡	Mouse phenotype
H^+/K^+ ATPase	Prilosec (gastroesophageal reflux disease)	$5,684.0	α-polypeptide KO: pH of gastric contents is close to neutral rather than 3.14; β-polypeptide KO stomachs are achlorhydric.
	Prevacid	$2,951.0	
	Takepron	$776.0	
	Pantozol	$609.0	
Histamine H_2 receptor	Gaster (gastroesophageal reflux disease)	$896.0	Induction of gastric acid secretion by histamine or gastrin is completely abolished.
	Zantac	$727.0	
Erythropoietin	Procrit (anemia)	$3,430.0	Failure to produce red blood cells, embryonic lethal.
	Epogen	$2,158.0	
Granulocyte-colony-stimulating factor	Neupogen (neutropenia)	$1,300.0	Deficiency in the total bone marrow cells, colony-forming haemopoietic cells, granulocytes and monocyte precursors in the bone marrow.

*Correlations between the best-selling drugs and the knockout (KO) mouse phenotype for drugs used to treat gastroesophageal reflux disease (GERD) and haematopoietic disorders. ‡US $ in millions.

Another target for GERD is the histamine H_2 receptor (HH_2R). Drugs such as Gaster and ranitidine (Zantac) are antagonists of this G-protein-coupled receptor and inhibit gastric-acid secretion. In contrast to the expected fivefold induction of acid secretion by histamine treatment, the induction of gastric-acid secretion by histamine or gastrin was abolished in the KO animals[13]. Basal pH of the gastric contents in *Hh_2r*-null mice was normal. The KO animals also showed hypertrophy of the glandular region of the stomach. In the case of GERD, mouse KOs have clearly been informative as to the function of the two most important targets.

Haematopoietic growth factors

Procrit and Epogen are recombinant forms of erythropoietin that stimulate red-blood-cell production and are used to treat anaemia. KO of erythropoietin or its receptor in mice resulted in identical embryonic lethal phenotypes, in which death occurred at embryonic day 13 (REF. 14) (TABLE 2). However, a further study of the embryos indicated that there was a failure to produce red blood cells and, more specifically, a failure of fetal liver ERYTHROPOIESIS. So although this is one of the rare cases of embryonic lethality in this group of targets, a basic histopathological analysis of the developing embryos quickly produced relevant information that indicated the gene function and pharmaceutical utility of the target.

Recombinant granulocyte-colony-stimulating factor (G-CSF; Neupogen) stimulates neutrophil production and is used to treat NEUTROPENIA. G-CSF$^{-/-}$ mice showed chronic neutropenia with a 70–80% reduction in circulating neutrophils[15]. The heterozygous animals had an intermediate phenotype, and had an approximately 30% reduction in circulating neutrophils. KO animals also had bone-marrow deficiencies, including reduced numbers of granulocyte, macrophage and blast progenitor cells. These results are in agreement with the known utility of Neupogen.

Immunological indications

Mouse genetics has also been productive for the evaluation of targets for immune modulation. The histamine H_1 receptor (HH_1R) is the target of a large family of antihistamines, including loratadine (Claritin), fexofenidine (Allegra) and cetrizine (Zyrtec). The KO mouse of this receptor results in a decreased responsiveness of the immune system[16,17] (TABLE 3). The proliferative response of *$Hh_1r^{-/-}$* splenic T cells treated with anti-CD3 antibody was reduced five to eightfold and the proliferative response of *$Hh_1r^{-/-}$* splenic B cells in response to anti-IgM was reduced three to fourfold compared with wild-type controls. When *$Hh_1r^{-/-}$* splenic T cells from ovalbumin-immunized mice were exposed to ovalbumin, there was a four to sixfold reduction in the proliferative response relative to wild-type T cells. Receptor-null mice treated with a T-cell-independent antigen produced about a ninefold lower titer of IgM, and the KO mice also displayed a significant reduction in IgG3 and IgM levels in response to immunization with the T-cell-dependent antigen ovalbumin. This decreased B- and T-lymphocyte responsiveness in the KO mice points to a role for Hh_1r in immune system function. Remarkably, many of the known side effects of the antihistamines, including altered alertness and activity levels, were also observed in the KO mice[18–20]. The KO mice displayed reduced exploratory behavior in a novel environment as measured by overall movement and rearing in the open-field test. The KO animals also had a less discernable circadian rhythm as measured by activity levels and a large decrease in activity in the dark phase. KO mice showed an increase in the latency of moving from the open to the closed arm in the elevated-plus-maize test, which indicates a decrease in anxiety. In the resident-intruder test, the mutant mice had a prolonged latency of attack as compared with wild-type mice, and the mutant mice also showed a decrease in nociception response in the late phase of the formalin paw test. This emphasizes the value of KO mouse studies for clarifying the on-target effects that might otherwise be mistakenly considered off-target side effects.

ERYTHROPOIESIS
Red blood cell development, in which a pluripotent stem cell produces, by a series of divisions, committed stem cells that give rise to cells that will divide only a few more times to produce mature erythrocytes.

NEUTROPENIA
A decrease in neutrophil numbers in the peripheral blood.

Table 3 | **Best-selling drugs and the KO-mouse phenotype: immunology***

Drug target	Drug name (utility)	2001 Sales‡	Mouse phenotype
COX2	Celebrex (arthritis) Vioxx	\$3,114.0 \$2,555.0	Reduced inflammation, significant reduction in collagen-induced arthritis, reduced febrile response and decreased polyp formation.
COX1 and COX2	Voltaren (arthritis)	\$631.0	COX1: decreased acute inflammation, decreased pain, decreased clotting, increased sensitivity to gastrointestinal damage, decreased polyp formation, see COX2.
Leukotriene receptor	Singulair (asthma)	\$1,375.0	Decreased extravasation in intraperitoneal zymosan challenge; 5-lipoxygenase KOs have decreased airway responsiveness in the ovalbumin challenge and reduced pulmonary fibrosis.
TNF-α	Enbrel (arthritis) Remicade	\$762.0 \$721.0	Decreased contact hypersensitivity and decreased IgG and IgE.
Histamine H_1 receptor	Claritin (allergy) Allegra Zyrtec	\$3,159.0 \$1,577.0 \$990.0	Decreased T- and B-cell response, decreased alertness and altered activity level.
Inosine monophosphate dehydrogenase 2	CellCept (transplant rejection)	\$625.0	Embryonic lethal; heterozygotes show significant impairment of T-cell activation and function.
Glucocorticoid receptor	Flovent (asthma) Advair Pulmicort Flonase	\$1,317.0 \$1,224.0 \$775.0 \$726.0	Null mutations result in early lethality; point mutation used to demonstrate role in inflammation.
Calcineurin	Sandimmun (transplant rejection)	\$1,083.0	KO of calcineurin Aβ exhibit reduced T cells in periphery, reduced thymocytes, defective lymphocyte activation and impaired allograft rejection.

*Correlations between the best-selling drugs and the knockout (KO) mouse phenotypes for drugs used for immune modulation, allergy, inflammation, arthritis and transplantation. ‡US \$ in millions.

Cyclooxygenase-1 and -2 (COX1 and COX2) are important mediators of inflammation and crucial targets for pharmaceutical intervention. The COX2-specific inhibitors, such as celecoxib (Celebrex) and rofecoxib (Vioxx), have achieved very large sales in the short time that they have been on the market. They are approved for the treatment of arthritis on the basis of their ability to decrease inflammation. The less selective non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium (Voltaren), aspirin and ibuprofen, target both the COX1 and COX2 isozymes. These general inhibitors are used to treat inflammation, pain, thrombosis and familial adenomatous polyposis.

The *Cox1* and *Cox2* KO mice have been studied extensively to determine the roles of COX1 and COX2 in the inflammatory process[21–25]. *Cox1*$^{-/-}$ mice have impaired platelet aggregation, as might be expected on the basis of the anti-thrombotic effects of NSAIDs such as aspirin[26]. *Cox1*$^{-/-}$ female mice have problems with parturition, which results in most offspring being born dead. There is roughly a 70% reduction in arachadonic-acid-induced skin edema in homozygotes, and an intermediate level of reduction in heterozygotes, compared with wild-type controls. *Cox1* mutants also produce about 25% less prostaglandin (PG) after carageenan treatment in the air-pouch model[27]. PGD_2 production in the first 2 hours after mast-cell activation is eliminated in *Cox1*$^{-/-}$ mast cells[28]. In spite of a greater than 90% reduction in basal-tissue PG production, there are no spontaneous gastrointestinal lesions observed. However, *Cox1*$^{-/-}$ mice are more susceptible to gastrointestinal tissue damage in response to ingestion of dextran sodium sulfate[29]. There is also a significant decrease in nociception as determined by a delayed response in the hot-plate assay, and a decreased response in the stretching assay in both homozygotes and heterozygotes[30]. Finally, anticancer effects are observed in the *Cox1*$^{-/-}$ and *Cox1*$^{+/-}$ mice. *Cox1*$^{-/-}$ mice have a 70% reduction in intestinal polyp formation on the *Min* background[31] and a 75% reduction in skin papillomas in the two-stage skin carcinogenesis model[32].

Cox2 KO mice are infertile due to impaired ovulation, implantation and DECIDUALIZATION[26,33]. Adult mice have kidney defects characterized by decreased numbers of, and poorly developed, glomeruli, dilated and atrophied renal tubules and they eventually succumb to end-stage renal disease. *Cox2*$^{-/-}$ mice have a reduced febrile response to lipopolysacharide[34]. In other tests of inflammation, *Cox2*$^{-/-}$ mice have a 75% reduction in the production of PG after CARRAGEENAN treatment in the air-pouch model[27], their mast cells do not produce late-stage PGE_2 after activation[28] and KO animals display decreased synovial inflammation and joint destruction in the collagen-induced arthritis model[35]. Mutation of *Cox2* also results in an anticancer effect as indicated by

DECIDUALIZATION
Formation of the deciduas, the inner layer of the wall of the uterus, which envelops the embryo, forms a part of the placenta and is discharged with it.

CARRAGEENAN
A sulphated cell-wall polysaccharide found in certain red algae, which contains repeating sulphated disaccharides of galactose, and sometimes anhydrogalactose, and is used to induce an inflammatory lesion when injected into experimental animals.

an 86% reduction in intestinal polyps in the *Apc* background[36] and a 70–80% reduction in the *Min* background[31]. In addition, *Cox2*$^{-/-}$ mice have a 75% reduction in papilloma formation in the two-stage skin carcinogenesis model[32].

In summary, the knockouts of *Cox1* and *Cox2* have helped define the roles of these two Cox isozymes in the inflammatory process and have provided other data indicating additional uses for the currently marketed COX inhibitors in areas such as cancer.

Leukotrienes are also potent mediators of inflammation derived from arachadonic acid. The leukotrienes play a major role in the pathophysiology of asthma, and leukotriene-receptor antagonists, such as montelukast sodium (Singulair), are used to treat asthma. KOs have been made for the enzyme 5-lipoxygenase (5-LO; encoded by *Alox5*), the committed step in leukotriene biosynthesis, and the cysteinyl leukotriene receptor 1 ($CysLT_1R$), and have showed the importance of both ligand and receptor in the inflammatory process. The ovalbumin airway-responsiveness assay, which measures the airway reactivity after antigen exposure, is often used as a model for asthma . In this assay, mice are sensitized by intraperitoneal injection of ovalbumin and are later challenged with aerosols of ovalbumin. In contrast to wild-type controls, 5-LO-deficient mice showed reduced cholinergic responsiveness, airway eosinophilia and immunoglobulin output in the airway-responsiveness assay[37]. Pulmonary fibrosis is a common result of untreated asthma and fibrosis can be induced in mice using bleomycin. *Alox5*-KO animals showed reduced pulmonary fibrosis with reduced histological collagen, reduced hydroxyproline levels and no increase in lung inflammatory cells after bleomycin treatment[38]. The *Alox5*-KO animals were also more resistant to platelet-activating-factor-induced endotoxic shock and had a decreased swelling response to arachadonic acid in the contact-hypersensitivity assay[39]. *CysLt*$_1$*r*$^{-/-}$ mice showed reduced plasma-protein extravasation in the zymosan-induced peritonitis model[40]. KO mice also displayed decreased plasma-protein extravasation during passive anaphylaxis mediated by IgE. Therefore the *Alox5* and *CysLt*$_1$*r* KOs have demonstrated the role of leukotrienes in inflammation and asthma.

Eternacept (Enbrel) and infliximab (Remicade) are two anti-inflammatory drugs used to treat rheumatoid arthritis. Both drugs have a similar method of action — that is, the blocking of tumour necrosis factor α (TNF-α). Eternacept is a recombinant fusion protein comprising the extracellular ligand-binding domain of the TNF receptor and the Fc portion of immunoglobulin (Ig), whereas infliximab is a recombinant anti-TNF-α antibody. *Tnf-α*$^{-/-}$ mice completely lack splenic primary B-cell follicles and are unable to form germinal centers[41,42]. They also have impaired humoral response to either T-cell-dependent or T-cell-independent antigens. These abnormalities can be measured by quantifying serum Ig levels. *Tnf-α*$^{-/-}$ mice have severely impaired IgG and IgE antibody responses. *Tnf-α*-null mice also show decreased contact hypersensitivity as measured in the oxazalone-contact hypersensitivity assay. These changes in inflammatory and antibody responses indicate the importantance of TNF-α in immune responses.

Mycophenolate (CellCept) is an immunosuppressive drug used to prevent transplant rejection. Mycophenolate inhibits inosine monophosphate dehydrogenase (IMDH), which is the rate-limiting enzyme in *de novo* guanine nucleotide biosynthesis. T and B cells are crucially dependent for their proliferation on this *de novo* pathway versus the salvage pathway, in contrast to other tissues. KO of the IMPDH II gene results in embryonic lethality[43]; however, heterozygotes show a decrease in T-cell-proliferative response after stimulation with anti-CD28 and anti-CD3 antibodies. This decrease is enhanced by a second mutation in the hypoxanthine guanine phosphoribosyl transferase (*Hprt*) gene that is required for the salvage pathway. Once again, the impairment of T-cell function correlates well with the known effects of the IMDH inhibitors.

The glucocorticoid receptor (GR) is the target of the gluco- and corticosteroids (for example, fluticasone propionate (Flovent and Flonase), fluticasone propionate and salmeterol (Advair), and budesonide (Pulmicort)), which are used to treat inflammation. These drugs bind to and activate the GR, which results in alterations in gene expression. KO of the GR results in perinatal lethality, which makes it difficult to study inflammatory response in these animals[44]. In this case, the null mutation was uninformative as to the role of the GR in inflammatory pathways. A subsequent point mutation was made and used to replace the endogenous GR[45]. This point mutation abolishes the DNA-binding function of the GR but the receptor maintains its protein–protein interaction capabilities. This mutant has been used to dissect the functions of the GR that are dependent on DNA-binding verses protein–protein interactions, and has been used to indicate that the anti-inflammatory function of the glucocorticoids is not dependent upon the DNA-binding function.

Cyclosporine (Sandimmun) is an immunosuppressant used to prevent transplant rejection and is thought to work by inhibiting calcineurin catalytic activity in lymphocytes by forming complexes with cyclophilins and FK506-binding proteins. The predominant calcineurin isoform in lymphocytes is calcineurin Aβ (CnAβ). KO of CnAβ results in a significant reduction in CD3-positive T-cells, a 75% and 64% reduction in CD4- and CD8-positive thymocytes, respectively, and a decrease in thymic cellularity[46]. *Ppp3cb* (also known as *CnAβ*)$^{-/-}$ splenocytes showed a significant reduction in proliferative response to stimulation by anti-CD3 antibodies or PMA/ionomycin. Most impressively, 42% of *Ppp3cb* KO mice were promiscuous for the development of allogeneic tumours, which demonstrates impaired allograft rejection compared with 0% for control mice.

Central nervous system and neurology

Neurology is a particularly challenging field for drug discovery because of the difficulty of translating the results of behavioral tests in mice to what might be of therapeutic value in humans. Fortunately, there is a

growing body of data concerning the KO phenotypes of known CNS drug targets, which makes it possible to predict how mouse behavioural phenotypes may correlate with human diseases such as depression. In addition, a growing number of pharmacological agents used to treat human neurological disorders have been tested in mice using the standard mouse behavioural tests, thereby benchmarking what behavioural changes might signify a therapeutic potential in humans.

The atypical antipsychosis drugs such as olanzapine (Zyprexa), risperidone (Risperdal) and quetiapine fumarate (Seroquel) are a more complicated story, as they target more than one receptor. These drugs act primarily on the dopamine and serotonin receptors, and to a lesser extent the histamine receptors. Although one cannot point to the KO of a single target in this case, the phenotypes of KOs of these receptors include effects on movement, activity, anxiety, alertness and other behavioral phenotypes that might have provided some clues as to potential neuropsychiatric utility for these targets[18,19,47–60] (TABLE 4). These KOs score in many of the standard mouse behavioural tests. More importantly, KOs of single members of these gene families may lead to information that can direct future drug development for the production of more specific inhibitors that could provide enhanced therapeutic value with minimization of side effects.

The serotonin transporter is the target for a large number of the selective serotonin-reuptake inhibitor (SSRI) class of drugs (for example, paroxetine HCl (Paxil), sertraline HCl (Zoloft), fluoxetine (Prozac), venlafaxine HCl (Effexor) and citalopram HBr (Celexa)) that are used to treat depression. We have knocked out this transporter and have observed altered open-field behavior (B. P. Zambrowicz and A. T. Sands, unpublished data). The open-field test is often used to indicate effects on anxiety and depression, and in our work results from this test direct us to do more detailed tests to uncover the potential of a target in the areas of anxiety and depression.

Bupropion HCl (Wellbutrin) is an antidepressant/anxiolytic that inhibits both the dopamine and noradrenaline transporters. Both of these transporters have been knocked out in mice[61,62]. Noradrenaline KO mice scored in two of the classic tests used to evaluate antidepressants. In the tail-suspension and forced-swim tests the mutant animals displayed increased struggle and swim times, respectively, which indicates an antidepressant effect. These animals also habituated more rapidly to a novel environment as measured by the open-field test. KO of the dopamine transporter resulted in animals with highly elevated spontaneous locomoter activity in the open-field test. KO animals also took longer to habituate to the open field test and were much more active than wild-type animals during both the light and dark cycles. So both dopamine and noradrenaline transporter KOs produce results suggesting potential value in the area of depression.

Zolpidem tartrate (Ambien and Stilnox) are two drugs used to treat insomnia, and they target and activate the GABA (γ-aminobutyric acid) receptor. The GABA receptor is important for inhibitory neurotransmission and this receptor is also the target of the benzodiazepines — the most common anxiolytic drugs. KO of the $GABA_A$ β_3 subunit resulted in reduced viability and the surviving animals showed hyperactivity and hyperresponsiveness to sensory stimuli[63]. These animals also were observed to spin in tight circles for long periods of time and they had decreased motor coordination. As expected, genetic antagonism of the GABA receptor produces a hyperactive phenotype which is the opposite of the sedative effects produced by GABA receptor agonists that are used as drugs to treat anxiety and sleep disorders.

In the area of pain, the μ-opioid receptor (Oprm) is the target of morphine and other analgesic drugs (fentanyl (Duragesic) and tramadol HCl (Ultram)), which act as agonists of the receptor. Not surprisingly, the *Oprm*-KO animals have an increased sensitivity to pain[64]. In the hot-plate assay, both heterozygotes and homozygotes displayed a decreased latency of response. KO mice also showed no response to morphine in the hot-plate assay. The *Oprm*-KO is another excellent example in which genetic inhibition of the target results in effects opposite to those of the agonist drugs used to treat pain.

Table 4 | **Best-selling drugs and KO-mouse phenotype: CNS and neurology**

Drug target	Drug name (utility)	2001 Sales*	Mouse phenotype
Serotonin transporter	Paxil (depression) Zoloft Prozac Effexor Celexa	$2,673.0 $2,366.0 $1,990.0 $1,542.0 $714.0	Altered open-field behaviour.
Dopamine, serotonin and histamine receptors	Zyprexa (psychosis) Risperdal Seroquel	$3,087.0 $1,845.0 $700.0	Multiple targets; however, related KOs display behavioural phenotypes (movement, activity and anxiety).
Dopamine and noradrenaline transporters	Wellbutrin (depression)	$931.0	Multiple targets; however, increased activity levels (dopamine transporter); increased struggle in tail suspension (noradrenaline transporter).
GABA receptor	Ambien (insomnia) Stilnox	$704.0 $902.0	Hyperactive, hyper-responsive.
μ-opioid receptor	Duragesic (pain) Ultram	$875.0 $601.0	Increased sensitivity to pain.

*US $ in millions.

Table 5 | **Best-selling drugs and KO-mouse phenotype: menopause, metabolism and hypertension***

Drug target	Drug name (utility)	2001 Sales‡	Mouse phenotype
Estrogen receptor	Premarin (menopause/ osteoporosis)	$2,074.0	Reproductive defects, reduced bone mineral density.
	Evista	$665.0	
	Nolvadex (breast)	$630.0	
Probably farnesyl diphosphate synthase	Fosamax (osteoporosis)	$1,760.0	Embryonic lethal; heterozygous males have increased bone mineral density.
	Aredia (hypercalcemia)	$752.0	
Unknown target, perhaps ACC2	Glucophage (diabetes)	$2,049.0	Anti-diabetic effects seen in ACC2 knockouts.
Insulin	Humulin/insulin (diabetes)	$1,061.0	No phenotype for KO of insulin I or insulin II; insulin-receptor-KO mice display hyperglycemia, ketoacidosis, increased triglyceride levels and fatty livers; 10% of heterozygotes develop diabetes.
	Humalog	$628.0	
PPAR-γ	Avandia (diabetes)	$1,018.0	Increased insulin sensitivity in heterozygotes; embryonic lethal homozygotes.
Lipases	Xenical (obesity)	$570.0	PLRP2, decreased fat absorption, carboxyl ester lipase reduced dietary cholesterol ester absorption.
ACE	Vasotec (hypertension)	$1,050.0	Low blood pressure.
	Prinivil	$1,260.0	
	Zestril	$1,097.0	
	Lotensin	$899.0	
	Tritace	$635.0	
	Accupril	$605.0	
Angiotensin receptor AT_1	Cozaar (hypertension)	$1,905.0	Low blood pressure.
	Diovan	$1,113.0	

*Correlations between the best-selling drugs and the knockout (KO) mouse phenotype for drugs used to treat menopause and osteoporosis, diabetes, metabolism and obesity, and hypertension. ‡US $ in millions.

Although making the transition from mouse behavioral phenotypes to human drug development remains challenging, KOs provide a powerful method for identifying novel mechanisms for modulating mammalian behavior. The ability to directly measure genetic effects on mammalian behavior, whether done as part of a genetic screen or to test a specific hypothesis, provides advantages in an area where few alternative methods for novel target identification exist.

Menopause and osteoporosis

The oestrogen receptors α and β (ER-α and ER-β) are the targets for the agonist drugs oestrogen (Premarin) and raloxifene HCl (Evista), which are used to treat the symptoms of menopause, including osteoporosis. These receptors are also the targets of anticancer antagonist drugs, such as the breast-cancer drug tamoxifen citrate (Nolvadex), a potent anti-oestrogen that causes the regression of established tumours. Appropriately, KO of these receptors resulted in both reproductive and bone effects (TABLE 5). KO of *Er-α* results in sterility for both males and females and complete absence of breast-tissue development[65,66]. In addition, both oogenesis and spermatogenesis are defective. An analysis of bone mineral content (BMC) using dual X-ray absorptiometry (DXA or DEXA) revealed a marked decrease in BMC in *Er-α -/-* males[67]. There was an approximately 20% decrease in BMC in total body as well as in regional measurements of vertebra and femur. A decrease in total areal bone mineral density was also observed in total body and femur of *Er-α*$^{-/-}$ mice, as was an approximately 20% decrease in BMC/body weight. *Er-β*$^{-/-}$ female mice are fertile but produce fewer and smaller litters, whereas males remain fertile[68]. These phenotypes correlate well with the uses of both ER agonists to fight the effects of menopause, including osteoporosis, and antagonist drugs to fight proliferation in breast cancer.

Another important class of drugs used to treat osteoporosis is the bisphosphanates such as alendronate sodium (Fosamax) and pamidronate disodium (Aredia), which act by inhibiting bone resorption by osteoclasts. Recent reports indicate bisphosphanates are potent and specific inhibitors of farnesyl diphosphate synthase and this may be the mechanism of drug action[69–72]. We have knocked out the farnesyl diphosphate synthase gene in mice and our preliminary data indicate that this mutation results in embryonic lethality, but that the heterozygous males have increased bone mineral density as measured by DEXA and bone microCT (B. P. Zambrowicz and A. T. Sands, unpublished data). This increased bone mineral density correlates well with the inhibition of osteoclast function that results from treatment with bisphosphanates.

Diabetes, metabolism and obesity

Glucophage (metformin) is an anti-diabetic drug with an unknown target. It is, however, known to decrease the levels of acetyl-CoA carboxylase 2 (ACAC2) activity in the liver and to induce fatty-acid oxidation[73]. By histopathological analysis, *Acac2*$^{-/-}$ mice had fewer lipid

Table 6 | **Best-selling drugs and KO-mouse phenotype: blood, skin and autonomic regulation***

Drug target	Drug name (utility)	2001 Sales‡	Mouse phenotype
$P2Y_{12}$ receptor	Plavix (atherosclerosis)	$1,350.0	Decreased platelet aggregation.
Factor X	Lovenox/Heparin (thrombosis)	$1,301.0	Neonatal death due to massive bleeding.
β-adrenoceptor	Serevent (asthma) Toprol (hypertension)	$929.0 $722.0	Complicated by multiple targets; however, target implicated for cardiovascular disease and KOs have defined the targets for β- blockers, impaired relaxation of heart; no data related to bronchodialation
Muscarinic receptor M_3	Detrol (overactive bladder)	$617.0	Increased urine retention in males.
Retinoic acid receptor	Accutane (acne)	$690.0	Gross development defects and early lethality.

*Correlations between the best-selling drugs and the knockout (KO) mouse phenotype for drugs used to treat blood coagulation and thrombosis, autonomic regulation and dermatology. ‡US $ in millions.

droplets in their livers than wild-type mice[74] (TABLE 5). A measurement of total lipid and triglycerides in the liver indicated a 20% drop in total lipid levels and an 80–90% drop in triglycerides. The *Acac2*$^{-/-}$ mice also had 20% lower glucose levels, 60% lower fatty-acid levels and 30% higher triglyceride levels in the blood. The *Acac2-/-* mice displayed increased fatty-acid oxidation and an examination of food intake and growth indicated that these mice ate 20–30% more food than wild-type mice, yet weighed 10% less and accumulated less fat. For instance, the epidydimal fat pad was reduced in weight by 50% in *Acac2*$^{-/-}$ mice relative to wild-type controls. Although still speculative, the KO phenotype, as well as data demonstrating the decrease in Acac2 activity and increase in fatty acid oxidation after metformin treatment, suggests that the inhibition of ACAC2 may be an important component of metformin action.

Insulin is used to control blood glucose levels in diabetes. Insulin is encoded by two genes in mice — *insulin I* (*InsI*) and *insulin II* (*InsII*) — and there is a single insulin receptor. KO of either *InsI* or *InsII* alone results in no discernable phenotype, but double mutants have an acute diabetic phenotype[75]. KO of the insulin receptor results in death within 7 days of birth[76,77]. The receptor-null animals have hyperglycemia, ketoacidosis, increased plasma triglyceride levels and decreased liver glycogen. Histopathological analysis reveals a fatty liver in mutant mice. In addition, 10% of the heterozygotes develop diabetes. Although loss of either insulin gene alone does not provide information about gene function, the double-KO or receptor-KO mice do help elucidate the function of insulin for glucose homeostasis. Additionally, two dominant missense mutations in the *InsII* gene (Mody and Akita) act in a dominant-negative fashion, most likely by interfering with normal heterodimer formation[78,79]. Both mutations result in mice with diabetic phenotypes that include hyperglycemia.

The thiazoladinediones such as rosiglitazone maleate (Avandia) are a new class of drugs, and are agonists of peroxisome-proliferator-activated receptor γ (PPARγ) that are used to increase insulin sensitivity in the treatment of type II diabetes. KO of *Pparγ* in mice results in embryonic lethality, but heterozygotes challenged on a high-fat diet display an anti-diabetic phenotype[80]. Heterozygotes had decreased levels of circulating insulin at time points taken during the glucose-tolerance test. Glucose clamps were used to demonstrate an increased rate of glucose disposal and a decreased rate of liver glucose production after insulin induction. Therefore, the *Pparγ* heterozygous mice have an insulin-sensitive phenotype. This is a paradoxical observation, as the thiazoladinediones are agonists and not antagonists of PPARγ. Perhaps this will require further studies to determine the mechanism of action of these drugs. However, the heterozygote phenotype would clearly direct one down the path for studying the role of PPARγ in insulin sensitivity and type II diabetes.

Orlistat (Xenical) is an anti-obesity drug that acts by inhibiting gastric and pancreatic lipases, which results in an inability to absorb dietary fats. Two lipases that have been knocked out in mice are pancreatic-lipase-related protein 2 (Pnliprp2)[81] and carboxyl ester lipase[82]. The *Pnliprp2* mutants have decreased body weight from day 4 to weaning, diarrhea and the fat content of their dried faeces is increased 10- to 15-fold, and has a high proportion of the fat as indigested di- and triglycerides. The carboxyl-ester-lipase-KO animals absorb only 50% of the cholesterol ester of wild-type mice. Both these KOs demonstrate the importantance of lipases in fat digestion and absorption.

Hypertension

KOs have been extremely useful in the field of hypertension. Two large classes of anti-hypertensives target two components of the renin/angiotensin pathway. These include the inhibitors of the angiotensin-converting enzyme (enalapril maleate (Vasotec), lisinopril (Prinivil and Zestril), benazepril HCl (Lotensin), ramipril (Tritace) and quinapril HCl (Accupril)) and the inhibitors of the angiotensin receptor AT_1 (Cozaar and Diovan). KO of either of these targets results in a significant decrease in resting blood pressure in mice[83–86] (TABLE 5). The receptor-null and heterozygous mice have a decrease in systolic blood pressure as measured by tail cuff of 24 and 12 mm of Hg, respectively. KO of the angiotensin-converting enzyme gene resulted in an

approximately 33 mm decrease in systolic blood pressure by tail cuff and one report indicated an intermediate effect in heterozygous male mice[86]. Heterozygote effects may demonstrate a target dosage effect and identify a rate-limiting step in a pathway. Such findings could identify targets that may be more amenable to drug discovery, as they would not require 100% small-molecule inhibition in order to provide a therapeutic effect and might have a greater therapeutic window.

Blood coagulation and thrombosis

The anti-thrombotic drug clopidogrel bisulfate (Plavix) targets the platelet $P2Y_{12}$ or adenosine receptor. This drug reduces platelet aggregation and is used to treat atherosclerosis. KO of the ADP receptor helped to define the actual target of this class of drugs[87] (TABLE 6). In addition, the KO mice have highly prolonged bleeding times and their platelets do not respond to ADP as one might anticipate. Likewise, enoxaparin sodium (Lovenox) and other heparins are used to inhibit thrombosis and act by inhibiting Factor Xa. KO of Factor X results in embryonic and neonatal death[88]. All animals die within 20 days of birth and death is in all cases due to massive bleeding. In both cases, KO phenotypes have demonstrated mechanism of action of anti-thrombotic drugs.

Autonomic regulation

Two drugs that target β-adrenoceptors are Serevent, a bronchodilator for the treatment of asthma, and Toprol, an antagonist that regulates vascular tone to treat hypertension. Both of these drugs act nonspecifically on several receptors, and as a result complicate the correlation between receptor KOs and drug action. However, the KOs of the β-adrenoceptor family members have been useful for defining the targets for some drugs acting on this class of receptors, and have given some indication of the β-adrenoceptor role in the cardiovascular system[89–101] (TABLE 6). We are unaware of any published reports directly studying bronchodilation/ restriction in receptor-KO mice.

The muscarinic M_3 receptor ($CHRM_3$) is a target of the drug tolterodine tartrate (Detrol), an antagonist used to treat overactive bladder. M_3-muscarinic-KO mice had larger pupils, reduced salivary response and urinary retention compared with wild-type mice[102]. This urinary retention was mild in females, and an increase in bladder diameter to 5.7 mm compared with 4.2 mm for wild-type control females was observed. The urinary retention was severe in males and resulted in distended bladders with a diameter of 12.3 mm compared with 5.4 mm for male wild-type controls. The retention also led to histopathological abnormalities, including thinning of the bladder wall due to the distension and some lymphocyte infiltrations. Hydronephrosis was also detected in some of the homozygous males. These findings are consistent with the known effects of Detrol.

Dermatology

Isotretinoin (Accutane) is a form of retinoic acid (RA) used to treat acne. There are three genes encoding the RA receptor (*RAR*-α, -β and -γ) and each encodes two isoforms. *Rar-α*$^{-/-}$ mice display reduced viability and 60% of the animals die within 24 hours of birth[103] (TABLE 6). Most are growth retarded and die within 2 months. Many have webbed fore- and hindlimbs. Those surviving past 2 months appear normal, but males are infertile and show abnormalities in spermatogenesis. *Rar-β*$^{-/-}$ mice, in contrast, are normal and fertile[104]. *Rarγ* mutation results in growth retardation, early lethality, male sterility, tracheal cartilage malformations and homeotic transformations of the skeleton[105]. A number of isoform-specific mutations have also been generated, but none of these have shed light upon the use of RA for the treatment of acne. However, the developmental defects and embryonic lethality associated with the KOs are consistent with prohibition of use of Accutane during pregnancy. In fact, one could extrapolate from this finding to conclude that embryonic lethality or developmental defects associated with a target should be taken as significant cautionary data alerting preclinical researchers to potential on-target toxicity issues related to pregnancy.

Oncology

Cancer is an area where many of the standard drugs are chemotherapies that act upon some of the basic housekeeping machinery of the cell to kill rapidly dividing cells. Indeed, most of the current drugs for cancer therapy directly target DNA, DNA metabolism, or mitosis and cell-cycle control points. Therefore, KO of many targets for oncology might be predicted to result in

Table 7 | **Best-selling drugs and KO-mouse phenotype: oncology**

Drug target	Drug name (utility)	2001 Sales*	Mouse phenotype
Topoisomerases	Camptosar (colorectal)	$613.0	Premature senescence.
Estrogen receptor	Premarin (menopause/ osteoporosis) Evista Nolvadex (breast)	$2,074.0 $665.0 $630.0	Reproductive defects, reduced bone mineral density.
Leutinizing-hormone-releasing hormone	Lupron (prostate) Leuplin (prostate) Zoladex (prostate)	$833.0 $689.0 $728.0	Leutinizing hormone receptor: hypogonadism and reduced steroidogenesis.
CD20	Rituxan (non-Hodgkin's lymphoma)	$819.0	Depletion of a subpopulation of B cells.

*US $ in millions.

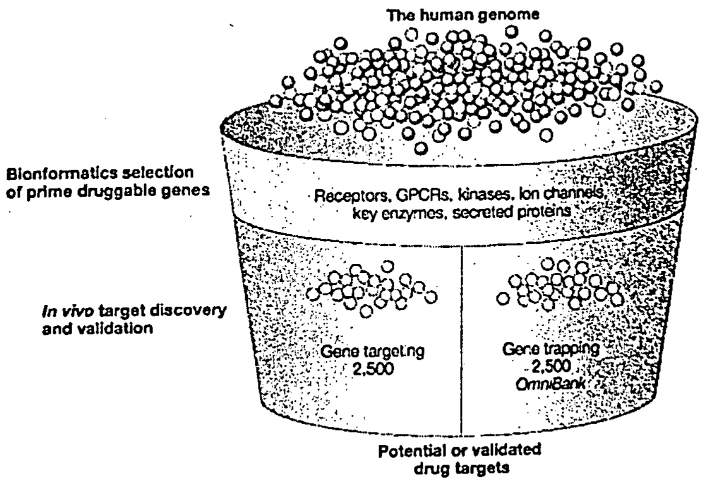

Figure 2 | **Knocking out the druggable genome: The Genome5000 Program.** This figure depicts the process of using bioinformatics to mine the druggable genes from the human genome, identify and knockout their mouse orthologs by gene targeting or gene trapping and analyze the resulting phenotypes to predict the best targets for drug discovery. GPCRs, G-protein-coupled receptors.

embryonic lethality or to exert global effects on cellular metabolism and organism growth. For instance, DNA-modifying agents and inhibitors of β-tubulin and TOPOISOMERASES are common chemotherapies that not only inhibit tumour growth, but also exert toxic effects on normal tissues that contain rapidly dividing cell populations. A number of drugs, such as irinotecan HCl (Camptosar), act by inhibiting DNA synthesis through the inhibition of topoisomerase. There are multiple topoisomerases and one that has been knocked out, topoisomerase IIIβ, results in animals with a lifespan that is decreased by about 50% relative to wild-type mice[106] (TABLE 7). These mice have pathological abnormalities in multiple tissues. These results suggest a role for topoisomerases in cell senescence and are in agreement with mutations in human topoisomerases that lead to premature senescence, such as that seen in WERNER'S SYNDROME. As cancer causes a loss in normal cell senescence, such a phenotype might direct one to develop drugs to drive cancer cells to senesce.

Certain cancer targets exert tissue-specific cell-cycle control based on the role of the targets in normal growth and development of particular tissue types within the body. The oestrogen receptor, for example, has already been discussed as a target for breast cancer. Another nuclear hormone receptor — leutinizing-releasing hormone receptor — is the target of anti-cancer drugs because of its normal role in gonadal development. Synthetic analogues of leutinizing-hormone-releasing hormone (LHRH), such as goserelin acetate (Zoladex), leuprolide (Lupron) and leuproline (Leuplin), are used to treat prostate cancer. Chronic LHRH treatment results in downregulation of leutenizing hormone (LH) release from the pituitary, and ultimately blocks testicular and ovarian steroidogenesis. LH binds to the LH receptor and KO of this receptor results in dramatically reduced growth and development of the reproductive tract and gonads[107]. This hypogonadism would also result in decreased testosterone production. Blockade of LH action for the treatment of prostate cancer makes sense relative to the KO phenotype.

Some of the latest, more specific, cancer targets are expressed only in or at a higher level in the cancerous cells (BCR-ABL in the case of imatinib mesvlate (Gleevec), ERBB2 (also known as HER2/neu) in the case of trastuzumab (Herceptin) and CD20 in the case of rituximab (Rituxan)). CD20 is the target of the rituximab antibody used to treat non-Hodgkin's lymphoma. KO of CD20 results in a depletion of a subpopulation of B lymphocytes[108].

TOPOISOMERASE
Enzymes that change the degree of supercoiling in DNA by cutting one or both strands.

WERNER'S SYNDROME
A disorder causing accelerated aging consisting of scleroderma-like skin changes, bilateral juvenile cataracts, progeria, hypogonadism, and diabetes mellitus; it results from the autosomal recessive inheritance of a mutation in a topoisomerase gene.

ORTHOLOGOUS GENE
Homologous gene in different species, the lineage of which derives from a common ancestral gene without gene duplication or horizontal transmission.

Going forward with reverse genetics

This retrospective study indicates that KO mice can be highly informative in the discovery of gene function and pharmaceutical utility for a drug target, as well as in the determination of the potential on-target side effects associated with a given target. It should not be surprising that gene function and physiology are so well conserved between mice and humans, as they are both mammals and contain similar numbers of genes, which are highly conserved between the species. It has recently been well documented, for example, that 98% of genes on mouse chromosome 16 have a human ORTHOLOG[109]. One might argue that some of the phenotypes were only found because the action of the drug was already known; however, that does not change the fact that the phenotypes were present and would have been readily detected by a well-designed phenotypic screen.

Mouse genetics is increasingly being used prospectively to identify the next targets for pharmaceutical discovery. Based on the literature reviewed here concerning the targets of marketed drugs, it seems obvious that these prospective studies are likely to provide a productive source of targets for future drug development. This is already happening and a number of drugs in pharmaceutical and biotechnology pipelines are being developed against targets whose function has been determined using mouse genetics. These targets include cathepsin K, melanin-concentrating hormone receptor (MCH_1-R), melanocortin receptors 3 and 4 (MC_3-R and MC_4-R), stearoyl-CoA desaturase and acetyl-CoA carboxylase 2 (ACC2). In the case of cathepsin K, KO mice for this protein have osteopetrosis due to defects in bone resorption[110] and small-molecule inhibitors are now being advanced for the treatment of osteoporosis. Likewise, *Mc_3-r*- and *Mc_4-r*-KO mice show obesity effects[111,112] and agonists of these receptors are being developed to treat obesity. Finally, *Mch_1-r*-KO mice are lean, hyperactive and hyperphagic[113,114] and *Acac2*[74] and stearoyl-CoA desaturase KO mice also have lean phenotypes[115]. Antagonists of MCH_1R and inhibitors of ACAC2 and stearoyl-CoA desaturase are being developed to treat obesity.

Knocking out the druggable genome

We have an ongoing five-year program to mine the top 5,000 potential drug targets from the human genome, to systematically knockout their mouse orthologs and

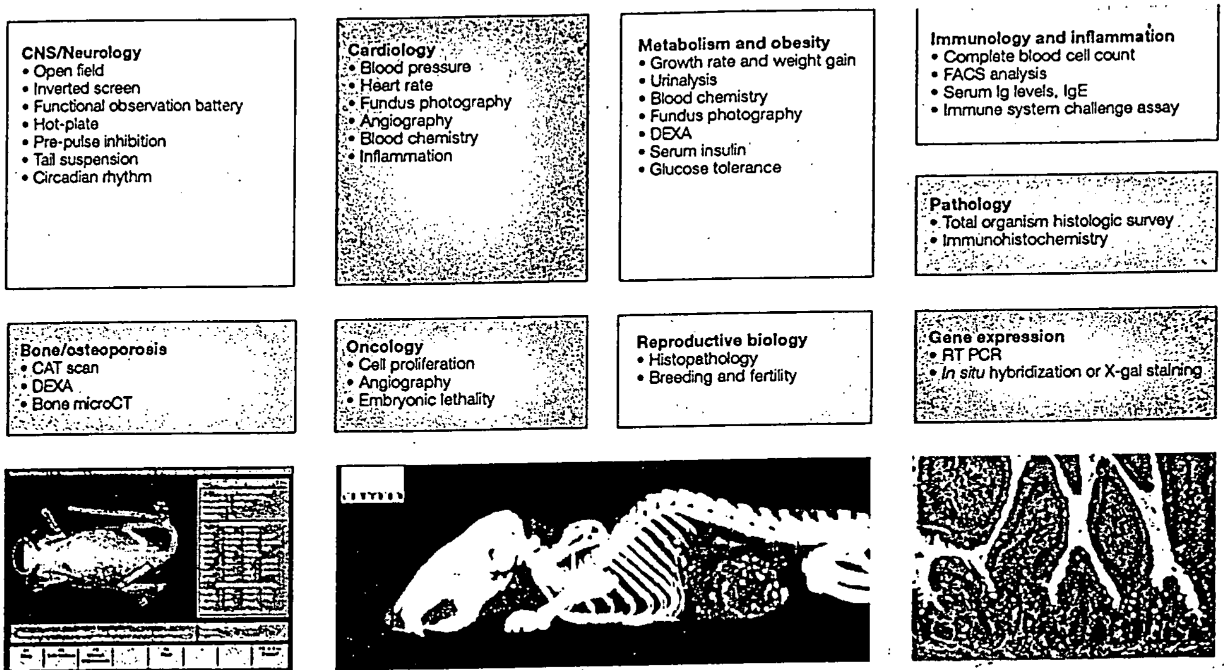

Figure 3 | **Comprehensive phenotypic screen for drug targets.** This figure summarizes the primary phenotypic screen that is carried out on all genes in the Genome5000 program. The screen was designed to identify new drug targets based on the knockout phenotypes of known drug targets and unmet medical needs. DEXA, dual energy X-ray absorptiometry; MicroCT, computed tomography; CAT, computer assisted tomography; FACS, fluorescence-activated cell sorting; Ig, immunoglobulin.

to determine their *in vivo* function in the context of mammalian physiology (FIG. 2). The scope of this program is sufficient to cover all secreted proteins as well as all members of the druggable gene families, such as GPCRs, ion channels, nuclear hormone receptors, proteases, phosphodiesterases, kinases, phosphatases and other key enzymes that together have been estimated to number as few as 3,051 genes[2]. After production of KO mice, a primary phenotypic screen is used to identify drug targets in cardiology, metabolism, immunology, neurology, psychiatry, ophthalmology, osteoporosis, reproductive biology and oncology (FIG. 3). Assays in the primary screen have been benchmarked with the administration of known drugs and carefully selected to be informative on the basis of knowledge of the phenotypes of known drug targets and unmet medical needs. The screen is designed to identify targets that meet three key criteria: (1) modulation of the target by a small molecule, antibody or therapeutic protein could provide significant therapeutic effect with minimal or no discernable on-target side effects; (2) the target represents a potential breakthrough for the treatment of disease with significant advantages over existing therapies; and (3) the program addresses a major unmet medical need associated with a large medical market.

Having now analyzed more than 750 potential targets *in vivo*, our experience suggests that after completing 5,000 'druggable' genes, 100–150 new, high-quality targets may be identified. This number is considerably more modest than the 5,000 to 10,000 targets suggested by some. However, given the number of targets currently used by the pharmaceutical industry and the low number of new targets commercialized each year, it appears much more realistic and represents at least a doubling of the number of targets currently fueling the pharmaceutical industry.

Summary

The data presented in this retrospective study of KOs of the top drug targets demonstrates the strong correlation that exists between phenotypes, mechanism of action and utility of associated therapeutics. It is clear that reverse genetics enabled by genome-wide KO technologies defines a path forward for the biopharmaceutical industry to discover the next generation of blockbuster therapeutics based on novel targets from the human genome.

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Lack of natural killer cell augmentation in vitro by human interferon gamma in a subset of patients with systemic sclerosis.

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Systemic sclerosis (SSc) is a generalized connective tissue disorder characterized by fibrosis of skin and various viscera. Natural killer (NK) cells are a subset of lymphocytes that can lyse targets without prior sensitization. Few studies have tried to assess NK cell function in patients with SSc. To evaluate NK cell cytotoxicity in patients with SSc and to see the extent of its augmentation in vitro by human interferon (hIFN) gamma in the clinical subset of limited and diffuse cutaneous diseases, we evaluated 27 patients with SSc and 22 age- and sex-matched controls by 51Cr release assay. Fifteen patients had limited cutaneous disease (mean disease duration 6.2 +/- 2.7 years) and 12 diffuse cutaneous disease (mean disease duration 5.7 +/- 2.4 years). Patients with limited SSc had significantly higher baseline NK cell function than controls ($p \leq 0.05$) and the augmentation following in vitro stimulation with hIFN gamma was negligible. Patients with diffuse SSc had lower baseline NK cell cytotoxicity than controls but this was not statistically significant. Augmentation with hIFN gamma in this group was comparable to controls. This study suggests that NK cells may have a role in the pathophysiology of this disease.

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Analysis of lymphocyte subpopulations in systemic sclerosis.

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Systemic sclerosis (SSc) is a chronic inflammatory connective-tissue disease of unknown etiology, characterized by fibrosis and microvascular injury in affected organs. It has become clear that the activated cellular-immune system plays a central role in the pathogenesis of SSc. This study analyzes the numbers of lymphocyte subpopulations and their relations with clinical and laboratory manifestations. We studied a group of 42 patients with SSc and a group of 28 matched normal controls by flow cytometry using the lymphocyte cell-surface markers CD2, CD3, CD4, CD8, CD19, CD25, CD45RA, CD56, CD71, HLA-DR, TCR alpha/beta, and TCR gamma/delta. Patients with SSc had similar percentages of CD2+, CD3+, CD3+ CD4+, CD3+, CD8+, CD25+, CD4+ CD45RA+, CD8+ CD45RA+, CD71+ cells, and CD4+/CD8+ cell ratio when compared to normal controls. In contrast, the percentages of TCR gamma/delta cells were significantly lower in SSc patients with diffuse and late-stage disease with pulmonary involvement, muscle involvement, and the presence of anti-Scl-70 antibodies. Patients with diffuse SSc in early- and late-stage disease had significantly increased percentages of HLA-DR in CD4+ and CD8+ cells. Patients with late-stage disease had increased percentages of CD4+ CD45RA+ T-cells. Patients with limited and early-stage disease had smaller percentages of B-cells (CD19+). Patients with diffuse and late-stage disease had smaller percentages of NK-cells (CD56+). These results suggest that T-, B-, and NK-cell alterations may be involved in the onset of the disease, and/or in the perpetuation of disease, and may eventually be useful as a prognostic indicator in selected patient subgroups.

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